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Quantitative study of wound infection in irradiated mice

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Bacterial infection of simple wounds was studied directly and quantitatively in adult mice given 6·5 Gy ^{60}Co. Three days later, when neutropenia was evident, the skin and the medial gluteus muscle of anaesthetized mice were incised. A suspension of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* or *Streptococcus pyogenes* was inoculated into the wound. Bacteria per mg muscle were enumerated 3, 4 or 7 days later. The geometric means of bacteria per mg were greater in irradiated than in non-irradiated mice. Phagocytic cells were present in the wounded tissue. Hence sublethal ionizing radiation enhanced the susceptibility of mice to infections of wounds by these four bacterial species.

1. Introduction

Sublethal ionizing γ-irradiation enhances susceptibility of animals to endogenous and exogenous bacterial infections by depressing normal haematopoiesis and host defences (Schechmeister and Bond 1951, Kaplan *et al.* 1952, Schechmeister *et al.* 1953, Clapper *et al.* 1954, Schechmeister 1954, Taliaferro *et al.* 1964, Anderson and Warner 1976). Neutropenia is a particularly important factor that predisposes to infection (Bodey 1985). Trauma, when superimposed on the consequences of irradiation, increases the chance of mortality (Ledney *et al.* 1985, Lindop *et al.* 1985, Rotblat 1986). Mice that were wounded 2 days after 9·0 Gy γ-radiation died sooner than those wounded immediately before or after irradiation (Ledney *et al.* 1985). Nuclear accidents may expose many persons to whole-body radiation with complex biomedical consequences, including infections (Finch 1987, Gale 1987 1988). Wounds that occur after exposure to radiation are likely to become infected in a neutropenic host. Enumeration of bacteria in a biopsy of infected wound tissue indicated severity of the infection and improved clinical judgement (Robson and Heggers 1969). Knowledge of the variety of bacteria that can cause enhanced post-irradiation wound infections is necessary to develop sound strategies for effective treatment of such combined injuries.

We developed a model to study bacteria directly and quantitatively at the site of infection after irradiation in order to study the efficacy of therapeutic agents in combined injured animals. After exposure of mice to a sublethal dose of γ-radiation we induced bacterial infections in simple wounds during neutropenia with minimal mortality for 7 days. The model was used to evaluate combined antibacterial therapies for infections by *Staphylococcus aureus* in irradiated mice (Brook and Elliott 1989). We present studies of four species of bacteria in this model, which

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show that wound infections by these bacteria were enhanced by prior sublethal ionizing radiation even when phagocytic cells were present near the wound.

2. Materials and methods

2.1. *Animals*

Approximately 400 female, B6D2F1/J mice (Jackson Laboratories, Bar Harbor, ME), 20–25 g, 8–19 weeks of age, were held in quarantine for 2 weeks. Representative samples were examined to assure the absence of specific intestinal bacteria and common murine diseases by microbiology, serology and histopathology. Up to nine mice were housed in sanitized 46 cm × 24 cm × 15 cm polycarbonate boxes with a filter cover (MicroIsolator, Lab Products, Inc., Maywood, NJ) on hardwood-chip bedding in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were given feed (Wayne Lab Blox, Continental Grain Co., Chicago, IL) and acidified (pH 2·5) water freely. The animal holding room was maintained with conditioned fresh air that was changed at least 10 times per hour at approximately 21°C and 50% (±10%) relative humidity and with a 12-h light/dark full-spectrum lighting cycle.

2.2. *Radiation*

The hair on the rear dorsal quarter of mice was shaved. Mice were exposed in perforated Plexiglas restrainers, which permitted normal exchange of air, to bilateral radiation from the AFRRI ^{60}Co source at a rate of 0·4 Gy/min at ambient temperature. The mid-line absorbed dose was 6·5 Gy. This sublethal dose was chosen to minimize mortality in infected mice. The lethal dose for 50% of uninfected B6D2F1/J female mice 30 days after exposure ($LD_{50/30}$) to this source is 9·65 ± 0·30 Gy.

2.3. *Leukocyte and thrombocyte counts*

Tail blood was drawn immediately before and daily after irradiation from five 15-week-old mice, selected randomly from a group of 10. Leukocytes and thrombocytes were counted microscopically by haemocytometer. The minimum number of detectable leukocytes was 110/μl.

2.4. *Bacteria*

Species of bacteria were selected because of their relative importance as causes of wound infections in human patients, because of their different effects on muscle tissue, and to provide diversity of the challenge. *S. aureus* and *Escherichia coli* are facultative species that are commonly isolated from wounds. *Klebsiella pneumoniae* provides a strong challenge because of its large polysaccharide capsule, whereas *Streptococcus pyogenes* causes necrosis of tissue, which may inhibit effective systemic treatment of the local infection.

S. aureus ATCC 25923, *S. pyogenes* AFRRI 4, *K. pneumoniae* AFRRI 7, and *E. coli* AFRRI 6 were transferred from stock suspensions in skimmed milk frozen at −20°C onto Columbia Sheep Blood (5%) Agar (SBA; BBL 21263, Cockeysville, MD). Cultures of *S. pyogenes* were incubated for 24 h in 5% CO_2 at 35°C, whereas the other species were incubated at 30°C for 24 h.

The highest number of bacteria of each species was used in inocula that would cause a serious infection but minimal mortality. These numbers were determined empirically. Preparation of inocula and counting of bacteria were previously described (Brook and Elliott 1989).

2.5. *Trauma*

Three days after irradiation, mice were anaesthetized by inhalation of methoxyflurane (Metofane™, Pitman-Moore, Washington Crossing, NJ). In initial experiments, shaved skin was wiped with a gauze sponge that was moistened with 70% ethanol and the skin over the right medial gluteus muscle was incised with a sterile no.15 scalpel. The incision was approximately 12 mm long and approximately 30° to the spine caudally. The exposed medial gluteus was then incised three times about 3 mm deep with the blade at different angles along the same stroke as the initial incision. To induce a specific infection, a 0·1 ml volume of a bacterial suspension in 0·9% NaCl was inoculated onto the incised muscle. The mice were placed in sanitized cages and observed until they recovered from the anaesthesia. Similar incisions were made in two irradiated and unirradiated mice without inoculation of bacteria. These mice were used to evaluate aseptic technique during removal of muscles.

Some inoculum occasionally leaked from the open wound, so the model was modified by injecting the inoculum s.c. to form a bleb over the right medial gluteus muscle. After the liquid was absorbed, in 20–30 min, the skin and muscle of anaesthetized mice were incised.

2.6. *Quantitation of bacteria in infected muscle*

Three mice from each experimental group were randomly selected and euthanized by cervical dislocation. The backs of the mice were wiped with 70% ethanol. The entire dorsal skin was removed and the entire medial gluteus, including abscess and necrotic tissue, was excised aseptically, weighed and homogenized. Homogenate and 100-fold dilutions were spread onto duplicate Columbia SBA plates.

After incubation, the number of bacteria per mg muscle was calculated (Brook and Elliott 1989). The mass of excised muscles in these experiments varied from 89 to 241 mg in unirradiated mice and from 114 to 300 mg in irradiated mice.

2.7. *Qualitative cultures of spleens and livers*

This was done as previously described (Brook and Elliott, 1989). The spleens and livers were removed aseptically and macerated with sterile cotton swabs in sterile Petri dishes. The tissues on the swabs were then spread onto SBA and incubated. Growth of bacteria was noted and identified presumptively.

2.8. *Histology*

We wanted to determine the local response of cellular defences in irradiated mice to a normally pyogenic infection. Strain ATCC 25923 of *S. aureus* is sensitive to penicillin, but is difficult to eradicate in wounded muscle (Brook and Elliott 1989). Ten mice were given 7·0 Gy γ-radiation, followed 3 days later with *S. aureus* and wounding. Three and 5 days later the wounded legs of five mice were dissected and fixed in formalin. Sections of wounds were examined microscopically

2.9. *Experimental design*

This study focused on severity of wound infection in irradiated and unirradiated mice with minimum mortality. Because some mice died, however, a sufficient number from one birth-date were included in each experiment to assure survivors for culture of bacteria. Tweny-four were irradiated, of which eight were held for measuring survival. Sixteen irradiated and 16 unirradiated mice were wounded and

bacteria were inoculated 3 days after irradiation. The mice were observed daily for general appearance, signs of local infection and signs of healing. Bacteria in the wounded muscle of three mice in each group were enumerated on the third or fourth and seventh days after bacteria were inoculated directly onto the wound, and on the fourth day in the modified procedure.

3. Results

3.1. *Leukocytes and thrombocytes in blood*

The ($\log_{10}$) number of leukocytes decreased immediately after irradiation from nearly $4{\cdot}0 \pm 3{\cdot}3\,\mu l^{-1}$ to daily averages between 2·3 and $2{\cdot}7\,\mu l^{-1}$ ($p < 0{\cdot}0001$), a reduction of 95–98%, between 3 and 13 days after irradiation (Figure 1). Recovery of the number of leukocytes began approximately 15 days after irradiation.

The ($\log_{10}$) number of thrombocytes remained stable (standard errors overlapped) for 5 days after irradiation at about $6{\cdot}2 \pm 5{\cdot}4\,\mu l^{-1}$, then decreased rapidly to $5{\cdot}9 \pm 4{\cdot}6\,\mu l^{-1}$, a reduction of 50% ($p = 0{\cdot}002$), on the sixth day after irradiation; the level was minimal between the eighth and tenth days (Figure 1), when the average was between $5{\cdot}1 \pm 4{\cdot}1\,\mu l^{-1}$ and $5{\cdot}3 \pm 4{\cdot}5\,\mu l^{-1}$, a reduction of 88–92%. Recovery of the number of thrombocytes began by the thirteenth day.

3.2. *Number of bacteria in infected muscles*

3.2.1. *Inoculation directly onto wounded muscle.* The number of the inoculated species increased by the third or fourth day above the number that was inoculated, except when *K. pneumoniae* was inoculated into unirradiated mice (Table 1). The number of all three species of bacteria in infected muscles was greater in irradiated mice than in non-irradiated mice 3 or 4 and 7 days after inoculation of bacteria and incision, but differences (d) on the seventh day between sets of irradiated versus non-irradiated mice infected by *S. pyogenes* ($\log_{10} d = 3{\cdot}61$) and *K. pneumoniae*

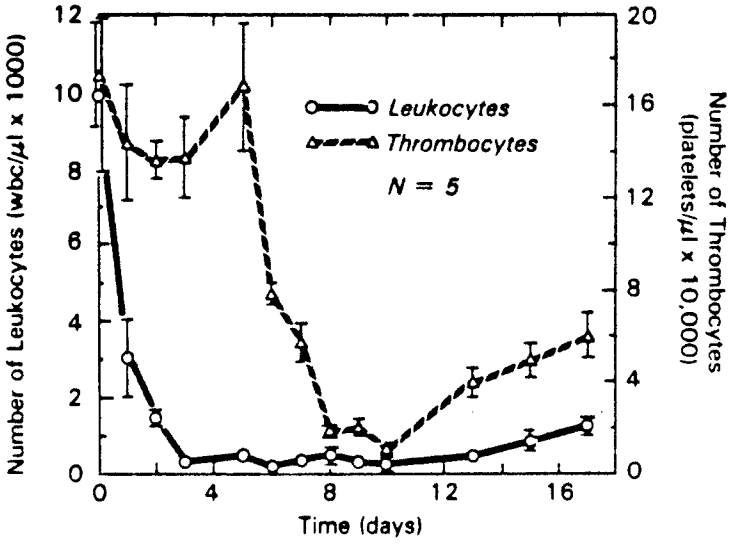

Figure 1. Numbers of leukocytes and thrombocytes in blood of mice exposed to 6·5 Gy ^{60}Co γ-rays. Blood was drawn from tail veins of five 15-week-old, female, B6D2F1 J mice that were chosen randomly from a group of 10.

Table 1. Numbers of bacteria in wounded muscles of unirradiated and irradiated mice.

Site of inoculation	Inoculum		Days after challenge	Log GM-CFU/mg (SEM)[a]			
	Species	CFU/mouse		Irradiated	Unirradiated	Difference[b]	p
Directly onto wound	*S. aureus*	$3{\cdot}7 \times 10^6$	4	6·68 (0·23)	5·45 (0·35)	1·23	0·042
	S. pyogenes	$9{\cdot}5 \times 10^2$	3	5·47 (0·14)	2·95 (0·68)	2·52	0·022
	K. pneumoniae	$1{\cdot}3 \times 10^2$	3	3·96 (1·69)	−1·63 (0·33)	5·59	0·032
Directly onto wound	*S. aureus*	$3{\cdot}7 \times 10^6$	7	7·34 (0·06)	3·31 (1·04)	4·03	0·061
	S. pyogenes	$9{\cdot}5 \times 10^2$	7	5·36 (0·36)	1·75 (1·99)	3·61	0·148
	K. pneumoniae	$1{\cdot}3 \times 10^2$	7	0·88 (1·67)	−1·97 (0·01)	2·85	0·230
Subcutaneous before wound	*S. aureus*	$3{\cdot}6 \times 10^6$	4	6·95 (0·05)	3·78 (0·97)	3·17	0·083
	S. pyogenes	$1{\cdot}1 \times 10^2$	4	5·70 (0·49)	4·31 (0·40)	1·39	0·093
	E. coli	$2{\cdot}0 \times 10^6$	4	6·91 (0·08)	1·26 (0·23)	5·65	0·000

[a] Logarithm, base 10, of geometric mean of CFU bacteria/mg muscle (standard error of the mean), $n = 3$.
[b] Irradiated − unirradiated.

($\log_{10}d=2{\cdot}85$) were not statistically significant because of high standard errors ($p>0{\cdot}1$, Table 1). In unirradiated controls, the concentration of inoculated species in wounded muscle decreased between 3 or 4 and 7 days after inoculation. In irradiated mice the concentration of *S. aureus* increased, *S. pyogenes* remained steady, and *K. pneumoniae* decreased between 4, 3 or 3 days, respectively, and 7 days. A species of *Micrococcaceae* was also isolated from muscles from six of six irradiated mice that were infected by *S. pyogenes*, but not from corresponding unirradiated mice or from irradiated mice infected by the other species. Inocula were not contaminated. Complete identification of this micro-organism was not performed. Experiments were repeated with similar results three times with *S. aureus*, once with *S. pyogenes* and once with *K. pneumoniae*.

3.2.2. *Subcutaneous inoculaton before wounding.* As in the previous experiments, when inocula of *E. coli*, *S. aureus*, or *S. pyogenes* were injected s.c. before the muscles were incised, the number of bacteria in wounded muscle was greater 4 days later in irradiated mice than in unirradiated mice ($p<0{\cdot}1$, Table 1). Experiments were repeated twice with *S. aureus* and once with *E. coli* with similar results at 7 days after challenge. A presumptive species of *Micrococcaceae* again was isolated (perhaps a species of *Staphylococcus* based on subsequent unpublished studies) together with *S. pyogenes* as occurred when bacteria were inoculated into the open wound. This consistent finding suggests that this stray species is a natural inhabitant of the skin and that there is a symbiotic affinity between these two species.

When the number of bacteria in muscles of irradiated versus unirradiated mice are compared, $p<0{\cdot}10$ at 3 or 4 days after challenge for all sets of data and at 7 days only for *S. aureus* that was inoculated directly onto the wounded muscle (Table 1). Variability in a set is evident from the standard errors of the means, which were smaller for irradiated than unirradiated mice in four of six sets.

Although the difference between numbers of *K. pneumoniae* in muscles of irradiated and unirradiated mice was the least statistically significant among the species ($p=0{\cdot}23$), the numbers of this species in muscles of irradiated mice 7 days after challenge were, nevertheless, higher than in those of unirradiated mice. In addition, although this study was designed to minimize mortality, a non-lethal infectious dose of *K. pneumoniae* in irradiated mice was difficult to achieve. Six of 12 irradiated mice that were challenged with *K. pneumoniae*, but not used for cultures, died during the 7 days after challenge, compared with two of nine challenged with *S. aureus* and none of those challenged with *S. pyogenes*. All mice that were only irradiated survived for 21 days of observation.

3.3. *Cultures of spleens and livers*

The inoculated bacterial species were detected in 9 of 18 spleens and 12 of 18 livers of irradiated mice, but not in those of unirradiated mice (Table 2). Only *K. pneumoniae* was not isolated from livers or spleens.

3.4. *Histology*

Lesions in mice that were irradiated, wounded and challenged with *S. aureus* showed focal loss of superficial epithelium and focally extensive coagulative necrosis from the dermis through the subcutis into underlying muscle. Bacteria colonized the area of necrosis and, in 5 of 10 specimens, along the fascial planes of

Table 2. Number of mice from which challenge bacteria were recovered from spleens and livers after combined injury.

Inoculum		Days after challenge	Irradiated : Unirradiated[a]	
Species	CFU/mouse		Spleen	Liver
S. aureus	$3{\cdot}7 \times 10^6$	7	2:0	3:0
S. aureus	$3{\cdot}6 \times 10^6$	4	2:0	3:0
S. pyogenes	$9{\cdot}5 \times 10^2$	7	2:0	2:0
S. pyogenes	$1{\cdot}1 \times 10^2$	4	1:0	2:0
K. pneumoniae	$1{\cdot}3 \times 10^2$	7	0:0	0:0
E. coli	$2{\cdot}0 \times 10^6$	4	2:0	2:0

[a] Three irradiated and three unirradiated mice were examined in each experiment.

the perimysial connective tissue. Inflammatory infiltrations of the subcutis or perimysium were adjacent to the areas of necrosis. Minimum to moderate numbers of degenerating neutrophils surrounded the zones of necrosis. Occasional healthy neutrophils penetrated into perimysium or endomysium. Macrophages were occasionally associated with areas of necrosis and often in nodular accumulations within the subcutis or perimysium adjacent to focal necrosis. Normal numbers of mast cells were within the subcutis, but occasionally in increased numbers within the areas of granulomatous inflammation. Mild to moderate proliferation of fibroblasts, multifocally in subcutis or randomly in perimysium, were seen adjacent to the area of necrosis, more on the fifth day after challenge and wounding than the third day.

3.5. *Observations of wound infections*

Although severity of muscle infections varied in irradiated mice, infections were generally more severe than those in unirradiated mice. Signs of infection, including swelling and excretion of pus, often remained in irradiated mice more than 7 days after bacterial challenge, longer than in unirradiated mice. However, spontaneous closing of the incision occurred in irradiated mice as well as in unirradiated mice between 7 and 14 days after challenge; however, muscles did not heal in irradiated mice.

Infection by each bacterial species caused a different effect on the muscle in irradiated mice. Infection is a process which includes effects of the host repsonses as well as effects of the micro-organism. A remarkable hardened and blanched disc of swollen muscle was often observed after inoculation of *S. pyogenes*; an abscess often formed after inoculation of *S. aureus*; dryness and erythema were observed after inoculation of *E. coli*; whereas clean serous lesions, sometimes with haemorrhage and oedema, and occasionally an abscess followed inoculation of *K. pneumoniae*. These effects were not noticeable in the unirradiated mice.

4. Discussion

This model demonstrated that a bacterial infection of a wound was more severe and persistent in an irradiated mouse than in an unirradiated mouse by quantitative measurement of the infecting bacteria even when neutrophils and macrophages were present; however, the neutrophils were degenerating and phagocytosis was not

observed. The numbers of bacteria that were recovered from muscles of irradiated mice were consistently higher than in unirradiated mice, although the differences between CFU in irradiated and unirradiated mice were more significant at 3 or 4 days than at 7 days after bacterial inoculation.

Although infection is a major complication in contaminated traumatic wounds (Gilbert *et al.* 1973, Pollock, 1988), septicaemia can overwhelm irradiated persons (LeRoy 1947), and surgery in irradiated tissue increases complications of wound healing (Luce 1984), clinical experience with bacterial infections in traumatic wounds in whole-body irradiated persons is limited and has received little attention. It is necessary to understand the mechanisms in order to manage effectively the infectious complications of combined injury (Walker and Conklin 1987). We are aware of no other experimental model used recently to study bacteria in wound infections in irradiated animals.

Kaplan *et al.* (1952) established that mice were most susceptible to bacterial infection of thighs by injection of a β-haemolytic streptococcus between 3 and 7 days after whole-body exposure of about 4·5 or 4·85 Gy X-irradiation. Histology showed liquefaction necrosis, avascularity and leukopenia in their irradiated mice. The bacteria disseminated into tissues sooner in irradiated than in unirradiated, infected controls; however, pressure induced by initial injection may have forced some bacteria into perimysial spaces.

The fact that the bacterial species in the inoculum, except *K. pneumoniae*, was detected in spleens and livers of most irradiated mice, but not at all in those of unirradiated mice, indicated bacteraemia and emphasized the decreased resistance and consequential systemic threat of wound infections in these irradiated animals, as was also shown by Kaplan *et al.* (1952). We have noticed that *K. pneumoniae* is often not isolated from livers and spleens of active mice after inoculation, but is always found in moribund mice. This observation indicates a rapid onset and course of septicaemia by this organism.

A satisfactory, complete explanation of changes that account for increased susceptibility to infections in irradiated animals (Kaplan *et al.* 1952, Schechmeister *et al.* 1952, Miller 1956) remains a challenge. Schechmeister *et al.* (1952) found that mice were susceptible to infection by an aerosol of *Streptococcus zooepidemicus*, and that leukopenia occurred between 3 and 21 days after a dose of about 3·5 Gy X-irradiation, but suggested that the increase in susceptibility could not be attributed to any one factor.

The local inflammatory process is important for control of infections, but radiation alters numbers and functions of phagocytic cells (Schechmeister 1954, Smith *et al.* 1963). Depression of this process probably contributes to mortality from septicaemia. Although neutrophils were attracted to the zones of necrosis and infection, they were degenerating and not noticeably phagocytic. Evidently, they can respond to chemotactic agents, but their phagocytic and cidal functions are deficient.

We selected the time of bacterial challenge to coincide with the precipitous decrease in the number of leukocytes 3 days after irradiation. When bacteria were inoculated into wounds 1 or 2 days after irradiation, infections were not as severe (Elliott and Brook unpublished data). Because granulocytopenia is similar in several mammalian species after 5–6 Gy X-irradiation (Patt and Maloney 1963), other mammalian species, e.g. humans, might be expected to show similar increased susceptibility to bacterial infections of wounds after sublethal irradiation.

Thrombocytopenia that occurred in our mice between the sixth and thirteenth days after irradiation is similar to observations in other animals (Cronkite 1946, Rosenthal and Benedek 1950, Cronkite *et al.* 1952). This depletion is caused by their response to deterioration of the vascular endothelium and exposure of collagen and by injury to bone marrow stem cells (Monroy 1987). Although all thrombocytopenic animals do not become purpuric (Cronkite *et al.* 1952), small local haemorrhages can occur in tissues during thrombocytopenia, which enable infecting bacteria to penetrate the tissue (Bond *et al.* 1954). The extravascular blood may also provide nutrients for bacterial growth, and thus enhance infection.

This model provides a practical means to study bacteria directly and quantitatively in irradiated, neutropenic animals, and is being used to determine the effect of combined antimicrobial therapies directly on bacteria *in vivo*.

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